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GIANT CLAMS IN A CHANGING OCEAN:

Effects of Ocean Warming and Acidification on *Tridacna maxima*, a Solar-powered Bivalve

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DISSERTAÇÃO DE **MESTRADO EM CIÊNCIAS DO MAR – RECURSOS MARINHOS**
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Dissertação de Candidatura ao grau de Mestre em Ciências do Mar – Recursos Marinhos submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

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During all this time they have guided and provided me with the tools to reach my dreams and never settle for least…

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With love,
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ABSTRACT

Giant clams (Cardidae; tridacninae) are iconic tropical bivalve molluscs enrolled in a rare endosymbiotic relationship with photosynthetic dinoflagellates from the genus *Symbiodinium* (zooxanthellae). This group portrays important ecologic roles while having a great cultural and economic value to local human communities. Nonetheless, there is a considerable knowledge gap regarding the effects of climate change, namely ocean warming and acidification, on such species.

The present dissertation represents a preliminary assessment of the physiological responses of juvenile *Tridacna maxima* clams, and respective symbionts, to the expected near-future conditions of warming (Δ + 3ºC) and high pCO_2 with concomitant acidification (Δ - 0.4 pH units). After a two-month acclimation period in a cross-factored design, an array of endpoints were evaluated: **(i)** respiration (R) and productivity (P), **(ii)** *Symbiodinium* histology, **(iii)** total haemocyte count (THC), **(iv)** heat shock response (HSR: HSP70/HSC70), **(v)** antioxidant enzymatic activities [catalase (CAT) and glutathione-S-transferase (GST)] and **(vi)** lipid peroxidation [malondialdehyde (MDA) levels].

The exposure to the experimental warming conditions elicited a decline in symbiont densities (associated with an increase in cellular sizes and asymmetry) and a decrease in the haemocytes numbers. There was no evidence of the activation of a heat shock response pathway and no detectable differences in antioxidant enzymatic activities. On the other hand, an increase in MDA levels, associated with cellular damage, was observed in the clams exposed to acidification.

Anthropogenic pressure has already been responsible for the decline of giant clam populations worldwide and climate change, particularly ocean warming, will most likely impose additional stress, undermining the conservation efforts taking place.

Keywords: Climate change; conservation; haemocytes; oxidative stress; photobiology; symbiosis; *Symbiodinium*;

RESUMO

Os bivalves da subfamília Tridacninae são invertebrados emblemáticos, típicos das regiões de recifes que estão envolvidos numa relação de simbiose com dinoflagelados fotossintéticos do género *Symbiodinium* (zooxanthelas). Para além da sua importância ecológica, têm também um grande valor económico e cultural para as regiões de onde provêm. Existe no entanto uma enorme falta de investigação que permita avaliar os potenciais impactos das alterações climáticas previstas para os próximos séculos podem ter neste grupo, nomeadamente subida de temperatura e acidificação dos oceanos.

A presente dissertação tem como objectivo avaliar as respostas destes holobiontes (juvenis de *Tridacna maxima* e respectivos simbiontes) às condições de aquecimento (Δ + 3ºC) e acidificação (Δ - 0.4 unidades de pH) expectáveis para a o final deste século. Após dois meses de exposição às condições experimentais foram avaliados uma séries de parâmetros : **(i)** respiração (R) e produção (P), **(ii)** condições celulares dos dinoflagelados **(iii)** contagem total dos hemócitos (THC), **(iv)** resposta ao choque térmico (HSR: HSP70/HSC70), **(v)** actividade de enzimas antioxidantes [catalase (CAT) e glutathione-S-transferase (GST)] e **(vi)** peroxidação lipídica [níveis de malondialdeído (MDA)].

A exposição às condições experimentais levou a um comprometimento do fitness dos holobionte, tendo sido evidente um declínio na população de simbiontes (associado a alterações de tamanho) e uma diminuição do numero de hemócitos. Não se detectou activação de resposta ao choque térmico nem alterações nos níveis de actividade das enzimas antioxidantes, pelo que aparentam ter sido insuficientes para lidar com as novas condições tendo sido evidente um elevado aumento do dano celular no animais expostos a condições de acidificação.

Encontrando-se já vulnerabilizado devido pressões antropogénicas, as alterações climáticas previstas para um futuro próximo vão muito provavelmente elevar os níveis de stress que assolam este grupo, colocando em causa dos esforços de conservação que têm vindo a ser postos em prática.

Palavras-chave: Alterações climáticas; acidificação dos oceanos; aquecimento global; stress oxidativo; simbiose; *Symbiodinium*; hemócitos; tridacninae;

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ABBREVIATIONS

CAT | Catalase

CDNB | 1-chloro-2,4-dinitrobenzene

GPP | Gross Primary Production

GSH | Glutathione

GST | Glutathione S-Transferase

H&E | Haematoxylin-Eosin coloration

HSP | Heat Shock Proteins (Stress Proteins)

HSR | Heat Shock Response

MDA | Malondialdehyde

NPP | Net Primary Production

OA | Ocean Acidification

P/R | Production to Respiration Ratio

R | Respiration

ROS | Reactive Oxygen Species

SB | Scale Bar

SOD | Superoxide Dismutase

SST | Sea Surface Temperatures

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INTRODUTION

1. INTRODUCTION

1.1. CHANGING PLANET

In the past 200 years, Man has become a distinct intervenient in the earth's climate. Since pre-industrial times, the atmospheric concentrations of greenhouse gases [such as carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O)] have increased to unprecedented levels in the last 800 000 years thousands of years. In particular, CO_2 concentrations have increased by 40 %, primarily due to the burning of fossil fuels and secondarily from changes in land use (IPCC, 2013). The current period, frequently termed the Anthropocene, has no direct analogue in the geological past (Riebesell, 2004; Brierley and Kingsford, 2009; Zalasiewicz et al., 2011).

The cumulative post-industrial CO_2 emissions have been affecting the heat balance of the earth and the carbonate equilibrium of the oceans. This has led to changes in the global water cycle, reduction and redistribution of snow and ice, global sea level rise, increase of climate extremes and alterations in the productivity on both land and oceans. According to the last IPCC report (2013), climate change is now unequivocal and, as the recent changes fail to be explained by natural factors, it is *extremely likely* that human pressure has been the dominant cause.

Demographic growth and changing life styles put an increasing pressure on energy production and, hence, man-made changes in the carbon cycle are expected to proceed. Predicted scenarios for climate change are expected to elicit major social and economic repercussions as both direct and indirect consequences of shifting environments (Stern, 2008; IPCC, 2013).

1.2. CHANGING OCEANS

Two thirds of our planet's surface is covered by oceans, which have been playing a vital and complex role in climate evolution and regulation since their formation (Bigg et al., 2003). Their buffer effect over the atmospheric temperature and their important role as sinks for gases, such as CO_2 (Bigg et al., 2003; Raven et al., 2005) is crucial to the planet's balance. They fostered the beginning of life and now host some of the most productive and biodiverse ecossystems on earth (Hughes et al., 2002; Brierley and Kingsford, 2009). Marine ecossystems provide a myriad of goods and services, on which the human society is both directly and indirectly dependent (Worm et al., 2006). Oceans worldwide, and particularly coastal areas, are already undergoing profound

transformations as a result of antropogenic pressure (Brierley and Kingsford, 2009; Cai et al., 2011; IPCC, 2013). Besides the potentially devastating ecological consequences, this will ultimately undermine human interests.

1.2.1. Ocean warming

The global ocean stores most of the energy present in the climate system, accounting for more than 90% of the energy accumulated in the past decades (IPCC, 2013). One of the main consequences of global warming is an increase in sea surface temperatures (SSTs) which presented a significant increase over the past 30 years at an average rate of 0.18 ± 0.16°C per decade. By the end of the century, SSTs are expected to rise by a further 1-4ºC. Furthermore, heat waves are expected to become more severe, frequent and last for longer periods in a warmer climate scenario (IPCC, 2013).

1.2.2. Ocean acidification

As anthropogenic emissions of CO_2 increase, continuous uptake by the oceans is changing the seawater chemistry. It is estimated that the oceans worldwide have absorbed approximately 30% of anthropogenic CO_2 emissions (IPCC, 2013). When the CO_2 partial pressure (pCO_2) in the atmosphere builds up in relation to the ocean's surface, carbon dioxide dissolves in the water and carbonic acid (H_2CO_3) is formed, further breaking up into hydrogen carbonate (HCO^{3-}). The latter reaction increases hydrogen ion (H^+) concentrations, thereby reducing the water's pH, in a process commonly referred to as "Ocean Acidification" (OA) (Raven et al., 2005; Fabry et al., 2008).

The increasing partial pressure of CO_2 in the ocean (known as hypercapnia) may result in a larger pH decrease over the coming centuries than in past 300 million years. Since the beginning of the Industrial era, there has been an average decrease in pH of 0.1 units. Due to the logarithmic nature of the pH scale, this may seem small, but actually accounts to a 30% increase in acidity and forecasts estimate a drop of 0.3-0.4 units in ocean pH by the end of this century (IPCC, 2013).

Moreover, many climate change models foresee that increasing atmospheric CO_2 under 'business-as-usual' scenarios will cause a decrease in calcium carbonate saturation in the sea over the next 100 years (Orr et al., 2005). The increasing amount of H^+ competes with Ca^{2+} ions to react and combine with the carbonate (CO_3^{2-}), producing a molecule of hydrogen carbonate (HCO^{3-}). This will reduce the amount of CO_3^{2-} available to produce calcium carbonate ($CaCO_3$), with adverse consequences to the calcification processes in marine organisms, particularly those with exoskeleton (Fabry et al., 2008).

1.2.3. Effects of ocean warming and acidification on marine biota

These unpreceded rates of physicochemical changes are likely to be followed by unpreceded impacts on marine organisms and ecosystems (Jackson, 2008; Brierley and Kingsford, 2009). Ocean warming and acidification have the potential to dramatically change the structure and function of marine ecosystems (Hoegh-Guldberg and Bruno, 2010; Doney et al., 2012). These environmental stressors may surpass organisms' tolerance limits, undermining the overall fitness and survival of the individuals and disrupting population dynamics (Pörtner, 2008). Indeed, rising temperatures are already affecting the abundance and distribution of many species, compromising the entire ecosystem (Perry et al., 2005; Brierley and Kingsford, 2009). Tropical ectotherms, in particular, are expected to experience severe consequences as, having evolved in relatively constant environments, possess limited acclimation capacities and tend to live closer to their thermal tolerance limits (Gilchrist, 1995; Hoegh-Guldberg et al., 2007).

On the other hand, besides effects in many other key biological traits such as metabolism (e.g.: Faleiro et al., 2015), reproduction (e.g.: Ross et al., 2011), behaviour (e.g.: Nilsson et al., 2012) and productivity (e.g.: Zimmerman et al., 1997), ocean acidification may be responsible for reduced calcification rates and dissolution of calcareous structures. Sessile and calcifying animals are expected to undergo more severe consequences (Kleypas, 1999; Riebesell et al., 2000; Hoegh-Guldberg et al., 2007).

While the isolated effects of both projected temperatures and CO_2 concentrations have been thoroughly studied over the past years, both stressors will act simultaneously in the future and the body of research for their synergistic action is still comparatively limited.

1.3. CORAL REEF ECOSSYSTEMS

Often described as the marine version of rainforests, coral reefs are among the most biologically diverse and productive ecosystems on earth, despite the oligotrophic nature of the surrounding waters (Lough, 2008). They provide a complex and varied habitat for near a third of the world marine fish among a vast array of other taxa, providing a wide range of social, ecological and economical goods and services (Moberg and Folke, 1999). Mainly through tourism, fisheries and coastal protection, estimates place coral reef's annual value to the global economy near the US $ 30 billion in net benefits (Cesar et al., 2003).

Figure 1 | Healthy coral reef on the North coast of East Timor (Photo by Nick Hobgood [CC BY-SA 3.0])

Unfortunately, coral reefs have been suffering a critical decline in the recent years with 27% considered permanently lost and another 30% at risk of extirpation by the year 2030. Increasing pollution and overexploitation represent the main causes of coral decline (Cesar et al., 2003). Moreover, coral reefs are considered exceptionally sensitive to global warming and ocean acidification. Beyond the overall impacts in species fitness, rising temperatures can lead to the emergence of new diseases and will, most likely, increase the frequency of mass bleaching events (generalized expulsion of the endosymbiotic dinoflagellates from the corals' tissues) (Hoegh-Guldberg, 1999). On the other hand, ocean acidification is expected to impair the calcification processes, compromising carbonate accretion and jeopardizing the reef infrastructure (Hoegh-Guldberg et al., 2007) . The social, economic and ecological implications of such processes can be devastating (Wilkinson, 1996; Cesar et al., 2003; Zeppel, 2011)

Understandably, most of the research regarding the impacts of climate change related stressors in this particular ecosystem is directly focused on corals, with other reef organisms receiving considerably less attention.

1.4. GIANT CLAMS

With colourful mantles and majestic sizes, giant clams (Cardiidae: Tridacninae) represent a highly emblematic, yet heavily targeted, bivalve group. Found throughout the tropical Indo-Pacific region (Othman et al., 2010), these animals have been living in association with coral reefs since at least the late Eocene (Harzhauser et al., 2008).

1.4.1. Taxonomy and diversity

The taxonomic placement of giant clams is a subject of much discussion, being classically placed in their own family, Tridacnidae (Knop, 1996) they were recently reclassified as a subfamily (Tridacninae) within the family Cardiidae (Hernawan, 2012). There are thirteen recognized extant species, including eleven species from the genus *Tridacna* and two of the genus *Hippopus* (Neo et al., 2015). Additionally, there are a number of extinct species which are conspicuous in the fossil record (Harzhauser et al., 2008). As the entire group is famous for their sizes, hence the name “giant clams”, *Tridacna gigas* is by far the largest species. Reaching over 1.30 m in length and 200 kg in weight (Knop, 1996), it constitutes the world’s largest bivalve (Yonge, 1975).

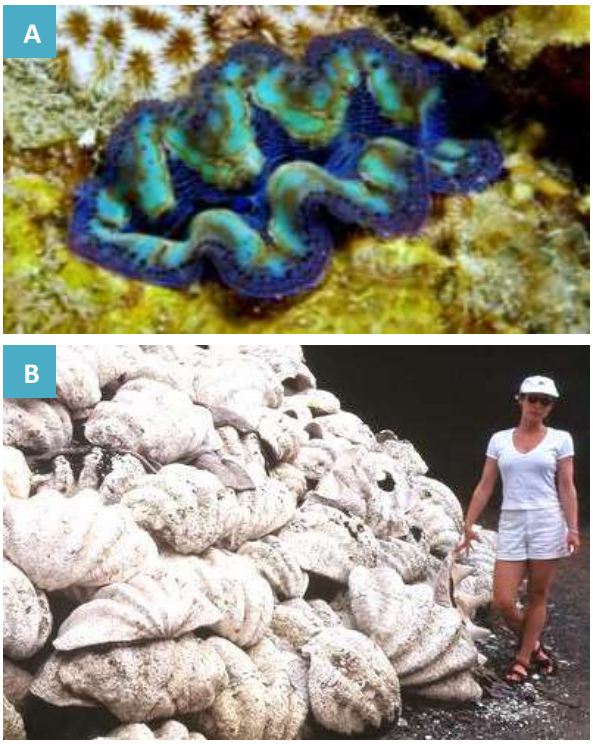

Figure 2 | (A) *Tridacna crocea*, exposing its colourful mantle (photo by Nick Hobgood [CC BY-SA 3.0]) **(B)** pile of *Tridacna gigas* shells (photo by David Hall [CC BY-NC 2.0])

1.4.2. Giant clams as holobionts

Their impressive growth rates, compared with other bivalves, are probably achieved due to the development of an endosymbiotic relationship with photosynthetic organisms, similar to the one seen in corals (Yonge, 1975; Knop, 1996; Klumpp, 1992). In fact, giant clams are mixotrophic bivalves (Klumpp, 1992; Yau and Fan, 2012), obtaining their nutrient requirements through both heterotrophic and photoautotrophic pathways. Along with the typical bivalve filter feeding and direct absorption of dissolved nutrients (Fankboner et

al., 1990; Fitt, 1993), they established an endosymbiotic relationship with dinoflagellate algae of the genus *Symbiodinium* (Baillie et al., 2000), commonly termed zooxanthellae (fig. 3). This symbiosis fulfils a major portion of the host nutritional and energetic requirements (Klumpp, 1992; Yau and Fan, 2012). The algae translocate part of their carbon-based photosynthetic outputs, such as glucose, glycerol, to the host (Fankboner, 1971; Ishikura et al., 1999; Muscatine and Cernichiari, 1969). Conversely, the clams provide their symbiotic partners with a homeostatic environment, protection against predation and excessive ultraviolet irradiation (Cowen, 1988; Ishikura et al., 1997), and most importantly, convey to the microalgae access to the CO_2 and nitrogenous wastes from their metabolism, fuelling the algae productivity (Fankboner et al., 1990; Fitt, 1993; Klumpp, 1992). The term *holobiont,* coined by Lynn Margulis, can be applied to this association, as clam and respective *Symbiodinium* live in symbiotic association for a significant portion of their life cycle (Margulis and Chapman, 1998; Weber and Medina, 2012).

By combining the clam opportunistic heterotrophy with the algae photosynthesis, this holobiont is able to thrive in the oligotrophic waters typical of tropical seas (Muscatine and Porter, 1977; Yellowlees et al., 2008) and even form reefs composed primarily of giant clams (Andréfouët et al., 2013). On the other hand, the strong light requirement imposed by the symbionts, restricts their habitat to clear, shallow waters (1 - 20 m) (Jantzen et al., 2008; Lucas, 1994).

In contrasts with hermatypic corals, in which zooxanthellae are reared intracellularly (Ambariyanto, 2002), giant clams have evolved a branched tubular system, spreading from the stomach to the exposed surface of

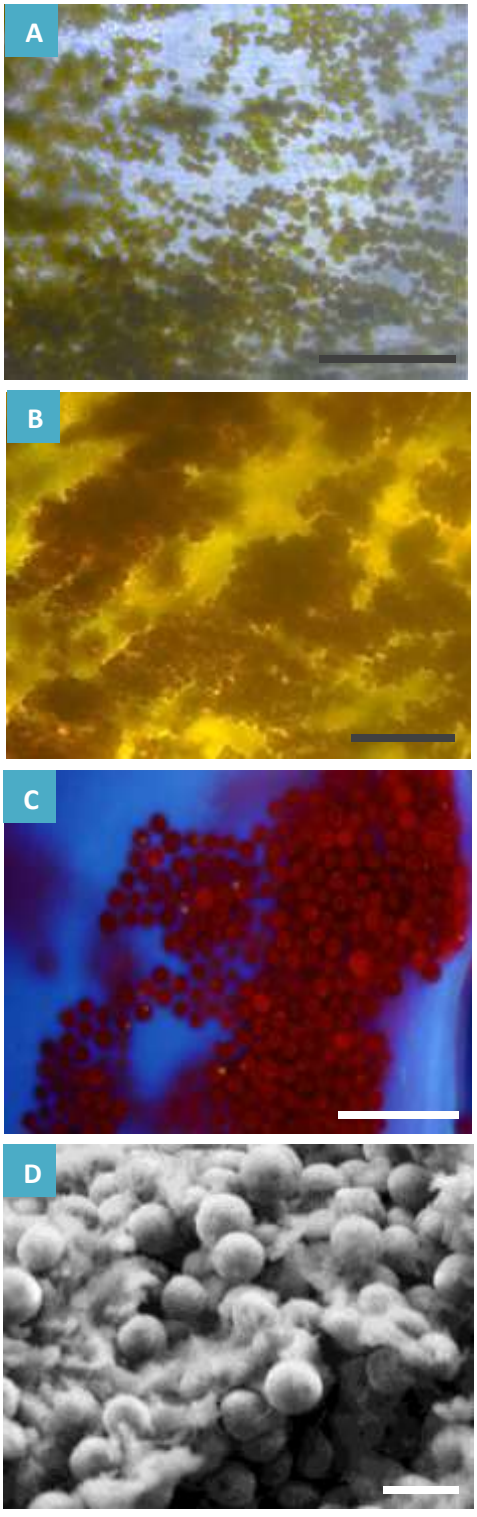

Figure 3 | Light micrographs of *T. maxima* mantle **(A)** showing the symbiotic dinoflagellates **(B)** stacked in the tubules of the channel network. SB=100 µm. **(C)** Autofluorescence of the mantle and symbionts from *T. maxima* under TRITC+DAPI filters. SB = 50 µm. **(D)** SEM micrographs of *Symbiodinium* spp. in *T. maxima*'s mantle. SB= 10 µm. (Photos: Own work)

the mantle. This channel network allows the penetration of light and providing stable microhabitats where their symbiotic partners can prosper (Hirose et al., 2006; Norton et al., 1992). These channels are pervaded by the host's haemolymph (Leggat et al. 2002), allowing the zooxanthellae to acquire nutrients both through the haemolymph or via the epithelium of the exposed mantle. The haemolymph, in turn, exchanges solutes with the seawater through the clam's gills (Yellowlees et al., 2008).

Figure 4 | *Corculum cardissa* shells (Photo by Rikaris [CC BY-SA 3.0])

In the case of giant clams, the symbiotic dinoflagellates are not vertically transmitted to the hosts' offspring (Jameson, 1976; Mies et al., 2012) and must be directly acquired from the environment, through ingestion, by the veliger larvae (Fitt and Trench, 1981; Hirose et al., 2006). It is noteworthy that, while constituting a rare association, giant clams are not the only bivalve molluscs living in symbiosis with microalgae (e.g.: *Corculum cardissa,* fig. 4) (Farmer et al., 2001).

1.4.3. Cultural, economic and ecological value

Giant clams are both an economically and culturally important resource throughout the Indo-Pacific Islands, and have been harvested since pre-historical times (Hviding, 1993; Leng and Bellwood, 1998). Their meat has been traditionally used as a subsistence food source and the shells are also used with both practical and decorative purposes (fig. 5) (Heslinga, 1996). More recently, their meat has become a delicacy (fig. 5A) and is even considered an aphrodisiac in some Asian and Pacific markets (Shang et al., 1991). The commercial trade of the more brightly coloured species for the aquarium industry is also a growing source of income (Bell et al., 1997). First introduced as a conservation effort to counteract the rapid decline in of wild populations (Heslinga and Fitt, 1987), giant clams farming and commercial hatcheries has become a profitable source of income in many tropical Pacific Island nations (Tisdell, 1992; Bell et al., 1997).

Figure 5 | *T. gigas* shell used as a holy water font in a Philippine chapel (Photo by Antonio Gil [CC BY-NC-SA 2.0])

Most of the research in this group's biology, which is now substantial, has been directed to the improvement of the aquaculture practices (Pearson and Munro, 1991; Hart et al.,

1998; Lucas, 2014). Much less attention has been focused on the ecological value of these organisms. As Neo et al. (2015) pointed out, in a recent review on this matter, giant clams play an array of important ecological roles in reef communities. They act as zooxanthellae reservoirs and contribute substantially to the reef productivity, topography, substrata and as shelter and food source for reef-associated organisms. Moreover, dense and healthy populations can potentially counteract eutrophication, both due to nutrient sequestering and their role as filter-feeders (Officer et al., 1982; Klumpp and Griffith, 1994; Neo et al., 2015).

1.4.4. Declining populations

Overexploitation have led to the decline of the wild stocks and giant clams are currently protected under Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) and listed in the IUCN Red List of Threatened Species. Conservation efforts are taking place (Guest et al., 2008; Heslinga, 2013), including the creation of legally protected sanctuaries (Andréfouët et al., 2013).

Despite conservation efforts, commercial harvesting, illegal trade and collection for the aquarium trade have led to the decline, and in some cases extirpations, of giant clams throughout their geographical range (Othman et al., 2010; Neo and Todd, 2012). Moreover, tridacnid clams suffer from the same habitat degradation associated with tropical reefs (Fabricius, 2005; Guest et al., 2008; Newman, 2015). Furthermore, they are also susceptible to stress-induced bleaching (expulsion of their symbiotic zooxanthellae) which is typically associated with increased sea surface temperatures (Norton et al., 1995; Grice, 1999).

1.4.5. Giant clams in a changing ocean

The cumulative action of acidification and elevated temperatures have been shown to reduce calcification (Rodolfo-Metalpa et al., 2011; Mackenzie et al., 2014), fertilisation and development (Kurihara et al., 2007; Parker et al., 2009), and growth and metabolism (Talmage and Gobler, 2011; Clark et al., 2013) in marine bivalve molluscs. Moreover, as a reef associated organism, the impacts of climate change in this group will most likely be amplified (Hoegh-Guldberg et al., 2007). Therefore climate change represents a challenge that may further undermine the recovery of these already vulnerable species.

Despite the considerable conservation efforts implemented to protect giant clams, the effects of climate change in this group are still very sparse. Most of the previous research has been based on short-term expositions (e.g.: Blidberg, 2000) which represent a useful

tool in the assessment of acute responses (Tomanek et al., 2011) but may not match the outcomes of chronical environmental stress (Clark et al., 2013). Recently, Watson et al. (2012) revealed a decrease in juvenile survival of *Tridacna squamosa* clams chronically exposed to the predicted future conditions of warming and high pCO_2 levels, however, survival rates were the only endpoint assessed and the physiological processes behind this response are yet to be understood. The synergistic effects of ocean acidification and global warming on giant clams are still widely unexplored and this knowledge gap limits the capacity to mitigate the impacts of global change on these species.

2. OBJECTIVES

The present dissertation represents a preliminary approach to the effects of projected ocean warming and acidification in a much emblematic, valuable and complex holobiont – the giant clam.

In this context, *Tridacna maxima* juveniles were reared for 60 days under one of four cross-factored experimental conditions, designed to reflect present-day conditions at the collection site and a future scenario of warming and acidification, based on IPCC predictions. At the end of the acclimation period we evaluated the effects of these climate change-related stressors on:

(i) Respiration (R), primary production (GPP) and P/R ratio;

(ii) *Symbiodinium* cellular densities, size and morphology;

(iii) Total haemocyte count (THC);

(iv) Heat shock response (HSR: HSP70/HSC70);

(v) Antioxidant enzymatic activity of catalase (CAT) and glutathione S-transferase (GST)

(vi) Lipid peroxidation [malondialdehyde (MDA) levels].

Through this approach we aim to better understand the eventual synergistic effects of ocean warming and acidification on these specific endpoints, and how this species may react to these different stress drivers.

MATERIAL AND METHODS

3. MATERIAL AND METHODS

3.1. Experimental setup and stocking conditions

3.1.1. Specimen acquisition and acclimation

Twenty juveniles of *Tridacna maxima* (fig. 11) were collected between January and February of 2015, from a local aquaculture, in the low lying atoll Ailinglaplap (Marshal Islands, Pacific Ocean, approximately 07°24′N 168°45′E) and transported through a commercial supplier (Tropical Marine Centre - Iberia). Each individual was placed in an artificial rock base, which the animal would use as a surface for attachment and support.

Figure 6 | *T. maxima* specimen with siphonal mantle exposed. **IS,** Incurrent syphon; **ES**, excurrent syphon; **P,** pinulla eyes.

Upon arrival to the aquaculture facilities of Laboratório Marítimo da Guia (LMG - Cascais, Portugal), the total 20 individuals (five replicates per treatment) were maintained in four 250-liter recirculating aquaculture systems (RAS). After a three week acclimation period to the new facilities, the parameters of each treatment were gradually adjusted to the experimental conditions, to which the individuals were exposed over the following 60 days.

3.1.2. Experimental design

The experimental design was chosen to reflect both present-day conditions at the region of collection (average ambient temperature of 28ºC and a pH of 8.0) and a future scenario, based on IPCC (2013) predictions for the end of the century. In the latter, increased temperatures (Δ +3ºC; 31ºC) and rising pCO_2 levels, with concomitant acidification (Δ -0.4 pH units; pH 7.6), are expected to act synergistically. Two additional treatments allow the isolation of the effects of increased temperatures and low pH. In summary, five individuals were reared in each of the four following treatments: **i)** 28ºC | pH 8.0 (Control); **ii)** 31ºC | pH 8.0 (Warming); **iii)** 28ºC | pH 7.6 (Acidification); **iv)** 31ºC | pH 7.6 (Warming + Acidification).

3.1.3. Life support systems

The life support systems (RAS) were previously filled with filtered (1 µm) and UV-irradiated seawater (salinity 35), with the tanks illuminated from above with two T5 lamps (24 Watts, one white and other blue actinic) under a photoperiod set to 12 hours of light and 12 of dark. Water quality was ensured using wet–dry filters (BioBalls), protein skimmers (Schuran, Jülich, Germany), fluidized sand bed filters (FSBF, TMC) and 30W UV-sterilizers (TMC, Chorleywood, UK). Ammonia, nitrites and nitrates concentrations were monitored regularly and kept below detection levels. Water changes of 20% were performed weekly to maintain total alkalinity and dissolved inorganic carbon speciation due to bacterial activity.

Temperature was kept stable by means of a water chiller (FRIMAR, Fernando Ribeiro Lda., Portugal) and submergible water heaters (Eheim, Germany). The pH values were adjusted automatically, via solenoid valves, with a Profilux controlling system (Kaiserslautern, Germany) connected to individual pH probes. pH values were monitored every two seconds and downregulated by injection of a certified CO_2 gas mixture (Air Liquide, Portugal) via air stones or upregulated by aerating the tanks using air-pumps (Hailea, China). Hysteresis ranges maintained pH levels at ± 0.05 margins. Additionally, seawater temperature and pH were manually controlled on a daily-basis (see table S1) using, respectively, a thermometer (TFX 430, EBRO) and a pH portable probe (SevenGo Pro, Mettler Toledo). Seawater carbonate system speciation was monitored spectrophometrically (595 nm) from total alkalinity according to (Sarazin et al., 1999). To fulfil the nutritional requirements of the species a plankton supplement (Pro-coral phyton, TMC) was added to the water daily.

3.2. Respiration and production

Oxygen consumption rates (µmol O_2 g^{-1} l^{-1} h^{-1}) were determined according to previously established methods (Rosa et al. 2012; Repolho et al. 2014). Each specimen was placed in an acrylic respirometry chamber (0.25 L) containing filtered (0.2 mm) and UV-irradiated seawater from each system, in order to avoid bacterial contaminations. Respirometers were immersed in a temperature controlled water bath (Lauda, Lauda-Königshofen, Germany) and allowed to acclimate for one hour. During the acclimation period, filtered seawater was pumped at a constant flow through the respirometers using water pumps (Eheim, Germany). Water-flow was then interrupted during one hour, and oxygen concentrations were recorded using Clarke-type O_2 electrodes connected to a multi-channel oxygen interface (model 928, Strathkelvin Instruments). Control chambers without animals were run simultaneously, to correct for potential bacterial respiration.

Two runs were made per individual, one exposed to light (same intensity as used during the 60-day acclimation) and other in complete darkness, to inhibit photosynthesis, taking into consideration the natural photoperiod of the animals. Before each run the electrodes were calibrated using oxygen-saturated seawater (using the correspondent maximum dissolved oxygen concentration value) and checked for electrode drift and microbial oxygen.

Respiration (R) of each holobiont was measured as the oxygen consumption rate (µmol O_2 g^{-1} l^{-1} h^{-1}) in complete darkness, while Net Primary Productivity (NPP) was obtained as the oxygen production rate (µmol O_2 g^{-1} l^{-1} h^{-1}) in the light exposed chambers. Gross Primary productivity (µmol O_2 g^{-1} l^{-1} h^{-1}) was calculated using the previous values (GPP = NPP+ R), under the assumption that respiration was constant in light and dark conditions. Production to Respiration ratios (P/R) were obtained by dividing the GPP by the R of each individual (Agrawal and Gopal, 2013; Baker et al., 2015).

3.3. Symbiont Histology

Histological studies were performed on small fragments dissected from the hypertrophied siphonal mantle and fixed overnight, at 4ºC, in a glutaraldehyde fixative solution (Merck, 2.5% in cacodylate buffer). Samples were then dehydrated in an ethanol gradient, cleared in xylene, and embedded in paraffin using a Shandon Citadel 2000 Tissue Processor. The fragments were inserted into histologic cassettes and included into paraffin blocks in a Shandon HistoCentre 2. Sections of 5-6 µm were made on a Leica RM2255 microtome and stained with standard haematoxylin-eosin (H&E) coloration (Hinzmann et al., 2013).

Histological sections were then observed and photographed using an Olympus DX 41 Microscope with a DP 70 camera. To estimate the density and size of symbiotic cells, a quantitative analysis was performed in three micrographs from three different mantle sections, per individual. Each micrograph was encrypted for individual and treatment and analysed using the freeware ImageJ. A 100 x 100 µm frame was randomly positioned in each micrograph demarking the area where symbiont cells were counted and measured. All the symbiotic dinoflagellates within the frame were counted to estimate population density and the results were then converted to symbiotic cells per square millimetre of siphonal mantle section (cells mm^{-2}). To estimate the size, diameter of the dinoflagellates (25 cells per micrograph) was measured twice, in an effort to achieve the largest and smallest value. The smaller value was subtracted to the largest to estimate the asymmetry and the average between both was used to calculate the area, reducing the error in the approximation to a circumference.

3.3. Total Haemocyte Count

Haemolymph was collected by carefully inserting a switchblade between the valves, disrupting the adductor muscle and tearing the mantle, to ensure the extraction of maximum volume. The fluid was passed through a funnel filled with glass fibre, in order to filter the larger particles, and collected in a falcon tube placed underneath. The haemolymph samples were kept on ice while processing and fixed using glutaraldehyde (Merck, 2.5% in cacodylate buffer) in a 1:1 ratio to the volume collected.

The total haemocyte count (THC) was obtained by observing the cells under a light microscope (BX 41 with digital camera DP70, Olympus, Tokyo, Japan) and counting them, using an improved Neubauer haemocytometer (Boeco, Hamburg, Germany). Three independent counts were performed per sample.

3.4. Biochemical Analyses

3.4.1. Preparation of tissue extracts

The individuals from each treatment were opened and their muscle tissue was collected and preserved at -80ºC until biochemical analyses were performed. The samples (100 mg wet tissue) were homogenized in 300 µL of Phosphate Buffered Saline solution (PBS, pH 7.3, consisting in 0.14 M NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl, and 1.47 mM KH_2PO_4), using an ULTRA-TURRAX® homogenizer (Ika, Germany). Each homogenate was then centrifuged for 20 minutes (14 000 x g, 4 °C). The supernatant fractions were collected and used to measure heat shock proteins (HSC70/HSP70 levels), catalase and glutathione S-transferase activities and lipid peroxidation (through MDA levels). The resulting values were standardised using the measurements of total protein content of the samples obtained according to the Bradford method (Bradford, 1976).

3.4.2. Heat Shock Response

HSP70 content (HSC70/HSP70) was assessed by ELISA (Enzyme-Linked Immunoabsorbent Assay), adapted from Njemini et al. (2005). A 10 µL aliquot of the supernatant was diluted in 250 µL of PBS. A 50 µL volume of each diluted sample was added to a 96-well microplate MICROLON®600 (Greiner Bio-One GmbH, Germany). The microplates were incubated overnight at 4 ºC. On the next day, the microplates were washed, four times, with 0.05 % PBS-Tween-20 and 100 µL of blocking solution (1 % Bovine Serum Albumin, BSA) was added to each well. The microplates were then incubated at room temperature, in the dark, for two hours. Afterwards, 50 µL of a solution with 5 µg mL^{-1} of primary antibody anti-HSP70/HSC70 (Acris, USA), that detects both 72 and 73 kDa proteins (corre-

sponding to the molecular mass of inducible HSP70 and constitutive HSC70, respectively), was added to each well. The plates were subsequently incubated at 37 ºC for a two-hour period. The microplates were washed once more, to remove the non-linked primary antibodies and 50 µL of secondary antibody [anti-rabbit IgG Fab specific, ALP conjugate (1 µg mL^{-1}) from Sigma-Aldrich (Germany)] prior to a new incubation period. After the washing process was repeated, 100 µL of substrate (p-nitrophenyl phosphate tablets, from Sigma-Aldrich, Germany) was added to each well and incubated for 30 minutes, at room temperature. Subsequently, 50 µL of stop solution (3 M NaOH) was added to each well, and the absorbance was read at 405 nm in a 96-well microplate reader (BIO-RAD, Benchmark, USA). The concentration of HSP70/HSC70 in the samples was calculated based on a standard curve of absorbance achieved through serial dilutions (from 0 to 2000 ng mL^{-1}) of purified HSP70 active protein (Acris, USA). The results are expressed in relation to the protein content of the samples (ng HSP70/HSC70 mg. $protein^{-1}$).

3.4.3. Catalase activity

Catalase (CAT) activity was assessed through and adaptation of the method described by Johansson and Borg (1988). In this assay, 20 µl of sample, 100 µl of 100 mM Potassium phosphate and 30 µl of methanol were added to a 96-well microplate, which was promptly shaken and incubated for 20 minutes. Afterwards, 30 µl of potassium hydroxide (10 M KOH) and 30 µl of purpald (34.2 mM in 0.5 M HCl) were added to each well, and the plate shaken and incubated for another 10 minutes. Subsequently, 10 µl of potassium periodate (65.2 mM in 0.5 M KOH) was added to each well and a final incubation was performed, for 5 minutes. Using a microplate reader (BIO-RAD, Benchmark, USA), enzymatic activity was determined spectrophotometrically at 540 nm. Formaldehyde concentration of the samples was calculated based on a calibration curve (from 0 to 75 µM formaldehyde), followed by the calculation of the CAT activity of each sample, were one unit of catalase is defined as the amount that will cause the formation of 1.0 nmol of formaldehyde per minute at 25ºC. The results are expressed in relation to total protein content (nmol min^{-1} mg^{-1}protein).

3.4.4. Glutathione S-Transferase activity

Total Glutathione S-Transferase (GST) activity was determined as described by Habig et al. (1974), measuring the formation of the conjugate of glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB). Aliquots (20 µL) from the supernatant of each sample were mixed in 180 µL of substrate solution (Dulbecco‘s Phosphate Buffered Saline with 200 mM L-glutathione reduced and 100 mM CDNB all from Sigma-Aldrich, Germany) and added to

96-well microplate. Using a microplate reader (BIO-RAD, Benchmark, USA), enzymatic activity was determined spectrophotometrically, recording the variance in absorbance per minute at 340 nm (determined using CDNB extinction coefficient of 0.0053 $\mu M^{-1}cm^{-1}$) for a total of six minutes.

GST activity is directly proportional to the increase in absorbance and can be estimated by means of the following equation:

$$\boldsymbol{GST\ activity}: \quad \frac{\Delta\, A240\ min^{-1}}{0.0053} \times \frac{total\ volume}{sample\ volume} \times dilution\ factor$$

The results are expressed in relation to the total protein content of the sample.

3.4.5. Lipid Peroxidation

Lipid peroxidation was estimated through the quantification of a specific end-product of the oxidative degradation process of lipids, the malondialdehyde (MDA) in an adaptation of the thiobarbituric acid reactive substances (TBARS) protocol (Mihara and Uchiyama, 1978).

Homogenates were treated with 8.1 % sodium dodecyl sulfate, 20 % trichloroacetic acid (pH 3.5), thiobarbituric acid and a 15:1 (v/v) mixture of n-butanol and pyridine. In this assay, the thiobarbituric acid and the MDA react, and produce a fluorescent product that can be detected spectrophotometrically at 532 nm. MDA concentrations were calculated using the Microplate Manager 4.0 software (BIO-RAD, USA), based on a calibration curve (eight concentrations, from 0 to 0.3 µM TBARS) made using MDA bis (dimethyl acetal; Merck, Switzerland). The results are expressed in relation to the protein content of the samples (nmol mg^{-1} protein).

3.5. Statistical Analyses

Two-way MANOVA (multivariate analysis of variance) were conducted in order to assess the effects of temperature and pH on related variables [metabolic parameters (R, GPP and P/R), zooxanthellae histology (density, size and asymmetry) and biochemical endpoints (HSR, enzymatic activity of CAT and GST)]. The results for the two-way MANOVA were interpreted according to Pillai's trace multivariate statistic, as it is often considered to be the most powerful and robust index (Johnson and Field, 1993). When significant effects were detected, follow-up two-way ANOVA were applied in order to discriminate further differences in each dependent variable, taking into consideration the results of the

MANOVA to achieve a better fitting model (inclusion of the interaction or simple main effects) and adjusting the significance level (α=0.05) with a Dunn–Šidák correction. In this case, the significance level was conservatively adjusted to 0.01 (two temperatures and two pH), order to protect against family-wise type I error. MDA content was analysed individually in a two-way ANOVA as its inclusion in the two-way MANOVA analysis (regarding biochemical parameters) would render a weaker model, due to a low correlation with the other dependent variables. THC was also analysed individually has it was obtained from a different tissue in an unrelated procedure. Normality and homocedasticity of the residuals were verified by Shapiro-Wilk and Levene tests, respectively. All statistical analyses were performed using IBM SPSS Statistics V. 21 (IBM, USA).

RESULTS

4.1. Survival, respiration and production

At the 29th day of acclimation period, one of the individuals exposed to the warming treatment died. For the rest of the acclimation period there were no more deaths to report.

The results regarding metabolic measurements are expressed in fig.7. The two-way MANOVA, showed no significant interaction ($F_{3,12}$ = 3.320, p = 0.057; Pillai's trace = 0.454) between temperature and pH on the combined dependent variables (R, GPP and P/R; fig.7). Moreover, neither temperature (two-way MANOVA: $F_{3,12}$ = 0.382, p = 0.768; Pillai's trace = 0.087) nor pH (two-way MANOVA: $F_{3,12}$ = 1.507, p = 0.263; Pillai's trace = 0.263) elicited a significant effect on these response variables.

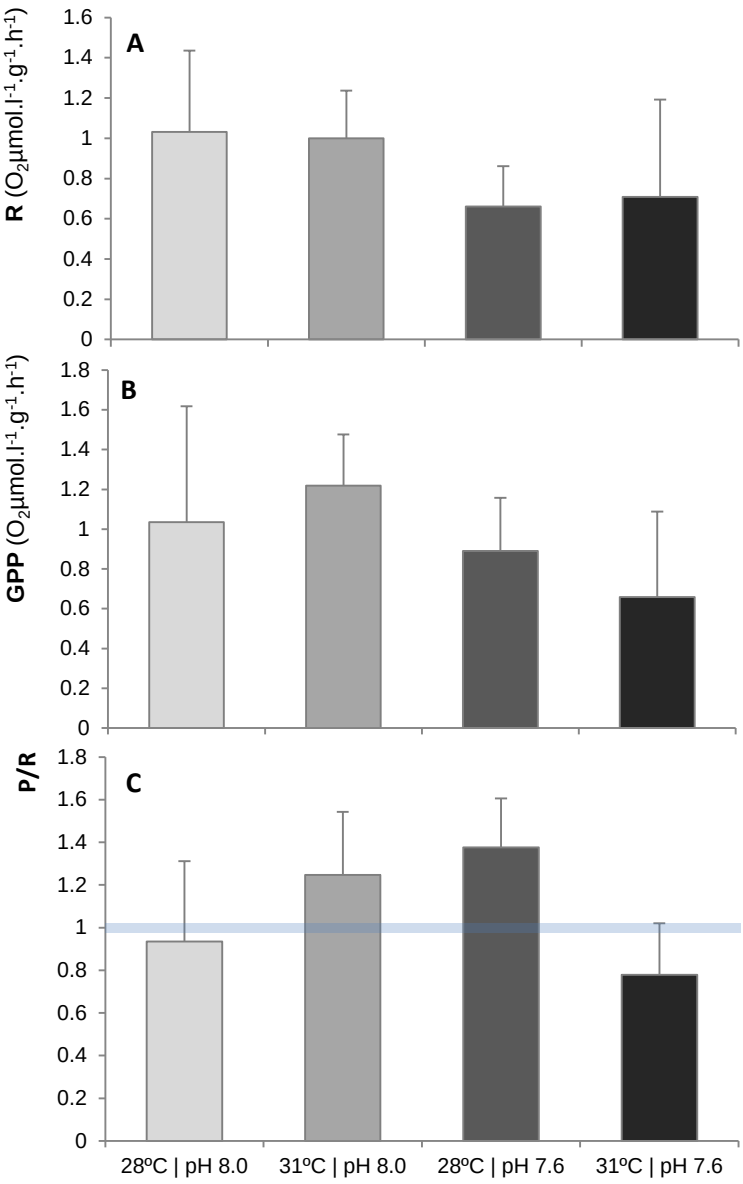

Figure 7 | Impacts of warming (Δ + 3°C) and acidification (Δ - 0.4 pH units) on **(A)** Respiration (R), **(B)** Gross Primary Productivity (GPP) and **(C)** Production to Respiration ratio (P/R) of the holobiont (*Tridacna maxima* clam and associated dinoflagellates). Values represent mean ± SD. Blue line refers to the compensation threshold.

4.2. Symbiont Histology

The observation of histological sections from the siphonal mantle revealed no significant interaction (two-way MANOVA: $F_{3,10}$ = 3.320, p = 0.057, Pillai's trace = 0.454) between temperature and pH on the combined dependent variables (population density and symbiont size and asymmetry; fig.8). Likewise, no effect of pH was detected (two-way MANOVA: $F_{3,10}$ = 2.877, p = 0.089, Pillai's trace = 0.874). There was, however, a significant effect of temperature (two-way MANOVA: $F_{3,10}$ = 23.182, p < 0.001; Pillai's trace = 0.874).

Regarding the effects of temperature, there was a significan decline (considering an adjusted α-level of 0.013) in *Symbiodinium* populations (two-way ANOVA: $F_{3,10}$ = 23.182, p = 0.001). Moreover, there was a significant increase in symbiont average size (two-way ANOVA: $F_{3,10}$ = 50.033, p < 0.001) and asymmetry with warmer temperatures (two-way ANOVA: $F_{3,10}$ = 52.886, p < 0.001).

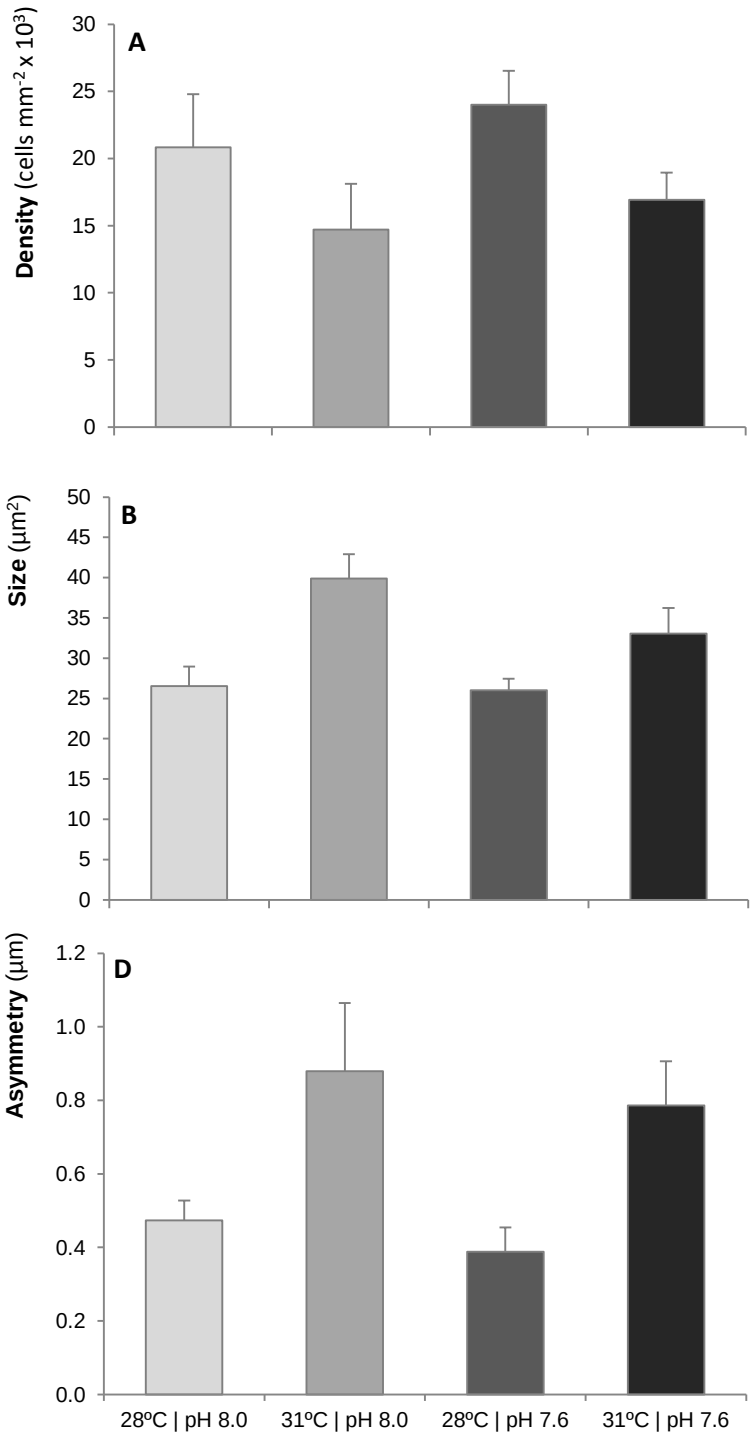

Figure 8 | Impacts of warming (Δ + 3°C) and acidification (Δ -0.4 pH units) on population density **(A),** individual size **(B)** and asymmetry **(C)** of the *Symbiodinium* from the mantle tissue of *Tridacna maxima*. Values represent mean ± SD.

4.3. Total Haemocyte Count

Temperature had a significant impact in the number of haemocytes (fig.S2) present in the haemolinph (fig.11; two-way ANOVA, $F_{1,15} = 6.526$, $p = 0.023$). Under higher temperatures (31ºC) there was a decline in haemocyte numbers compared to control temperatures (28ºC). On the other hand, there was no significant effect of pH (two-way ANOVA, $F_{1,15} = 2.297$, $p = 0.150$) nor significant interaction between both stressors.

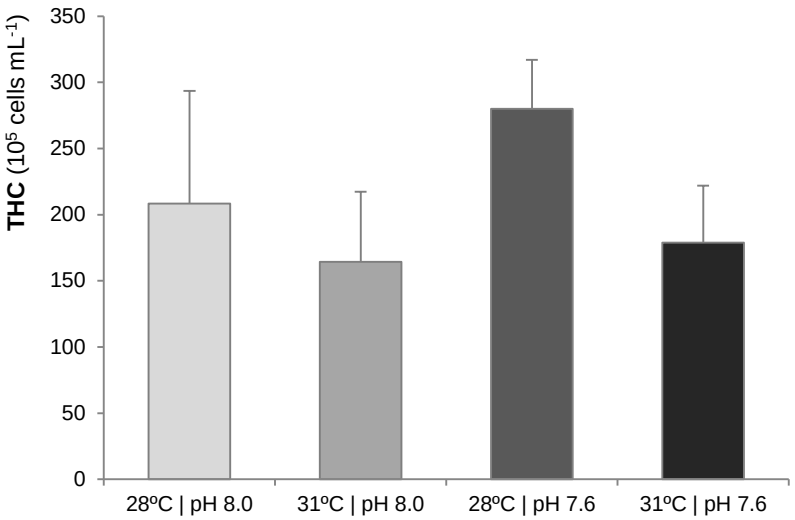

Figure 9 | Impacts of warming (Δ + 3°C) and acidification (Δ - 0.4 pH units) on total haemocyte count (THC) of *Tridacna maxima* juveniles. Values represent mean ± SD.

4.4. Biochemical analyses

Neither temperature (two-way MANOVA: $F_{3,11} = 1.220$, $p = 0.349$, Pillai's trace = 0.250) nor pH (two-way MANOVA: $F_{3,11} = 0.326$, $p = 0.807$, Pillai's trace = 0.082) had a significant effect over the combined response of heat shock proteins (fig.12), and enzymatic activity of CAT (fig.13a) and GST activity (fig.13b). Likewise, there was no interaction between both stressors (two-way MANOVA: $F_{3,11} = 0.597$, $p = 0.630$, Pillai's trace = 0.140). Regarding lipid peroxidation, there was a significant increase in MDA content with acidification (fig.13c; two-way ANOVA, $F_{1,15} = 6.082$, $p = 0.026$), while no effects of temperature (two-way ANOVA, $F_{1,15} = 0.158$, $p = 0.026$) or interaction between factors was detected (two-way ANOVA, $F_{1,15} = 0.958$, $p = 0.343$).

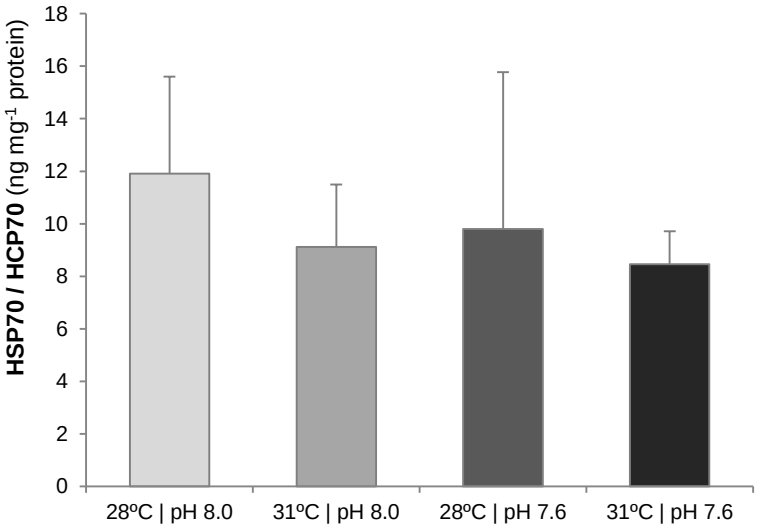

Figure 10 | Impacts of warming (Δ + 3°C) and acidification (Δ - 0.4 pH units) on total haemocyte count (THC) of *Tridacna maxima* juveniles. Values represent mean ± SD.

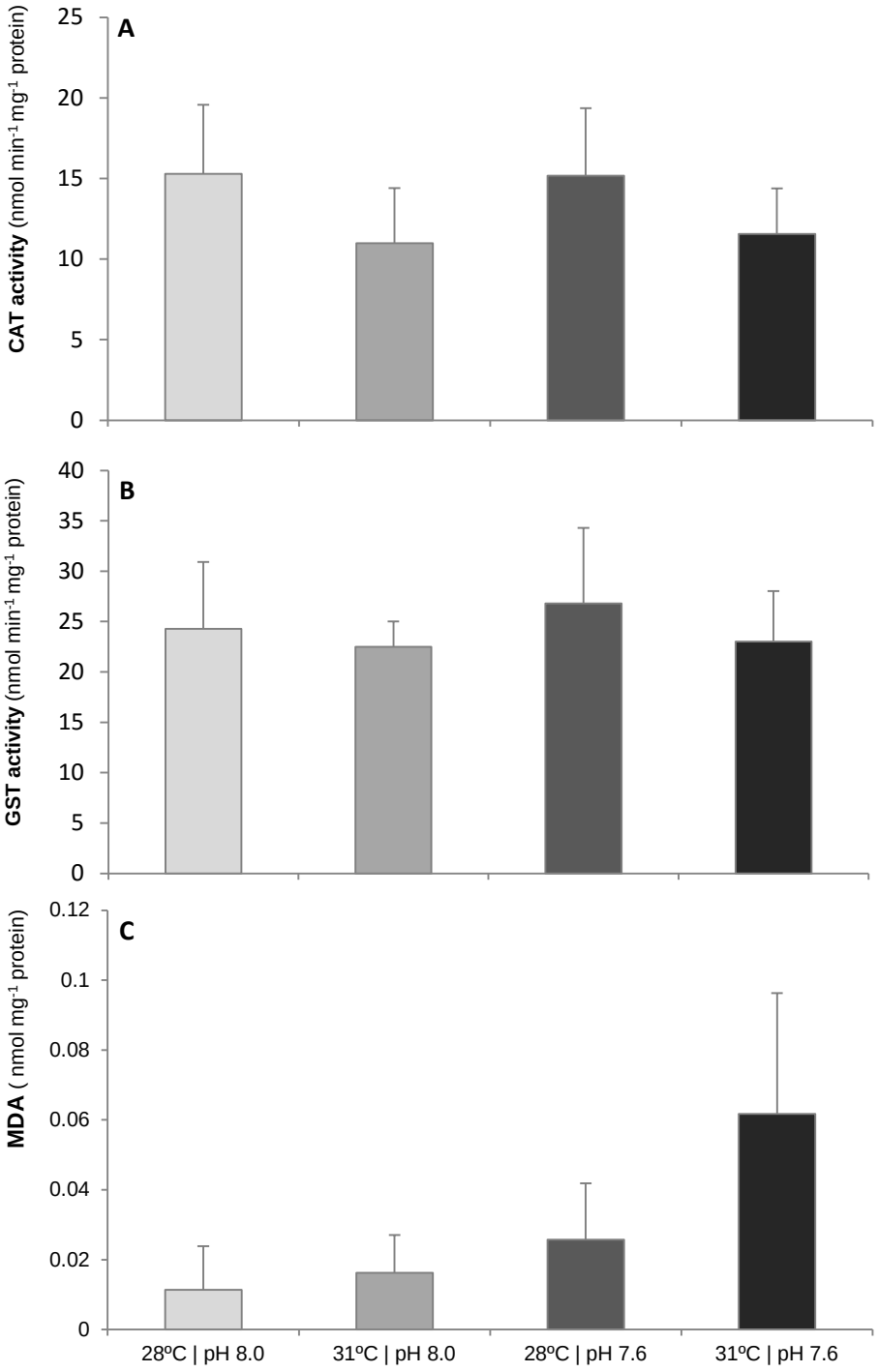

Figure 11 | Impacts of warming (Δ + 3°C) and acidification (Δ -0.4 pH units) on population density **(A),** individual size **(B)** and asymmetry **(C)** of the *Symbiodinium* from the mantle tissue of *Tridacna maxima*. Values represent mean ± SD.

DISCUSSION

5. DISCUSSION

5.1. Respiration and production

Temperature in the surrounding environment is known to directly affect the metabolic rates. Respiration, and other metabolic processes such as photosynthesis, tends to increase with temperature, due to a rise in the kinetic energy available for chemical reactions, until the optimal temperature is achieved. Beyond that point, metabolism declines again (Davison, 1991; Pörtner and Farrell, 2008). Routine metabolic rates have been found to increase in many ectothermic animals exposed to future warming conditions (e.g.: Aurélio et al., 2013) while some, due to the surpass of their optimal temperature see a decline in their metabolic rates (Blidberg et al., 2000). pH, on the other hand, has been found to further constrain the thermal tolerance window within which organisms are able to fully perform (Lannig et al., 2010; Faleiro et al., 2015).

In the present study, however, neither temperature nor pH had a significant effect on metabolic responses (both respiration and primary production). The experimental variation of temperature (Δ + 3ºC) and pH (Δ - 0.4 pH units) used in this study, aiming to reproduce the conditions foreseen for the near-future, may be too small to render a detectable response, especially considering the high individual variation and low sample size. Regarding respiration, a similar pattern has, nonetheless, been obtained by Reynaud et al. (2003), in a similar study with scleractinian corals where the temperature variation was also + 3ºC. Moreover, in what regards the effects of ocean acidification in primary production, this is in line with previous studies (Kroeker et al., 2013). It is noteworthy that the effects of acute warming in both R, GPP and P/R were previously evaluated by Blidberg et al. (2000) in several giant clam species (*Hippoppus hippoppus, Tridacna gigas, Tridacna derasa* and *Tridacna squamosa*) and, while acute and chronic effects may differ greatly, different responses were found between species, suggesting that the responses of R and GPP in these organisms to increasing temperature, and potentially other environmental stressors such as acidification, may be species-specific.

Although the observed results should be mostly explained by the clam and respective symbiotic dinoflagellates, it is worth noting that other small organisms, such as small fouling polychaete (fig.S1; see supplementary materials), which live in close association with the clam and were present in the chamber, may have accounted for a small percentage of the oxygen consumption.

5.2. Symbiont Histology

Like many other reef organisms, including corals, giant clams live in symbiosis with dinoflagellates which are key to fulfil the clam's energy demands and represent a vital adaptation to life in oligotrophic waters (Muscatine and Porter, 1977; Klumpp et al. 1992). Symbiont densities, besides their conditions and genetic makeup, can affect the host resistance against changing environmental conditions (Rowan and Knowlton, 1995; Jones and Berkelmans, 2010; Cunning and Baker, 2013; Maboloc et al., 2015).

In this experiment, the population density of *Symbiodinium* present in the clams' siphonal mantle was reduced under warmer conditions. Indeed, although complete loss of coloration was not observed in any animal during this study, the individuals from warming treatments presented a slightly more faded coloration by the end of the experiment (fig.S2; supplementary materials). This decline may be a natural response to warmer temperatures (Fitt et al., 2000) and may, as well, be indicative an approximation to the critical temperature for the induction of bleaching, a phenomenon characterized by the mass expulsion of the symbiotic microalgae (and loss of pigmentation) by the host, increasing the susceptibility of the holobiont (Ward et al., 2000). As a result of global climate change, the frequency and scale of bleaching occurrences have been increasing over the past decades (Hoegh-Guldberg, 2007). Despite the reported resilience of tridacna clams towards this phenomenon, mass bleaching events in these organisms have been previously recorded in association with a rise in sea surface temperatures (Gomez and Mingoa-Licuanan, 1998; Resources, 2013). The adaptive character of such phenomenon has been hypothesised, allowing the recolonization of the host with new symbionts, more fit to the new conditions (Buddemeier and Fautin, 1993). Furthermore, Cunning and Baker (2013) provided indications that the reduction of symbiont densities in corals under warmer conditions may also have an adaptive character, reducing the susceptibility to bleaching.

The decline in zooxanthellae numbers was accompanied by an increase in size and asymmetry of the cells, as the presence of enlarged cells was evident across the mantle tissue of heat exposed clams. The range of non-motile coccoid cell (the most common form exhibited by these symbionts in the hosts' tissue) sizes were in accordance with Stat et al. (2006). Differences in size and asymmetry may be due to different stages of the symbiont life cycle stages, possibly corresponding to either older, degenerative, cells or different reproductive strategies (Freudenthal, 1962; Fitt W. K., 1983; Maboloc et al.,2015). Alternatively, these enlarged cells may belong to a different, potentially more heat resistant, *Symbiodinium* strain or clade (Stat et al., 2006; DeBoer et al., 2012;

Deschaseaux et al., 2014; Jones and Berkelmans, 2010) that was able to thrive under the new conditions. Ultimately, nonetheless, changes in the symbiont conditions may impact the holobiont balance and its response to biotic and abiotic factors (Vennet al., 2008).

5.3. Total Haemocyte Count

Haemocytes are the main cellular components of bivalves' haemolymph and have a preponderant role in a wide range of homeostatic functions such as transport of oxygen and nutrients, immune responses, neuroendocrine regulation, shell biomineralization, detoxification and tissue regeneration (Bayne et al., 1979; Cheng, 1984). Moreover, the haemolymph of giant clams has a crucial role in its relationship with its symbionts, representing the immediate source of nutrients for the zooxanthellae (Fitt et al., 1995; Nakayama et al., 1997).

Haemocytes are crucial in physiological adaptation to changing environmental conditions (Cheng, 1984) and yet, the synergistic effects of warming and acidification on this cellular component are still poorly understood. The numbers and composition of haemocyte populations are highly dynamic and dependent on both the internal status of the individual and environmental stressors. The haematological parameters of bivalves reflect fairly objectively the general physiological and immunological status of the bivalves (Anisimova, 2014).

In the present study, temperature had a negative effect on the total number of haemocytes. Multiple studies with other species have pointed in a different direction, with haemocyte numbers increasing with temperature (Chu et al., 1993; Fisher et al., 1996; Liu et al, 2004). Mainly attributed to a rise in metabolism (not observed in this experiment), some authors have hypothesised this to be a coping response to the new conditions, due to the rise in pathogenic agents that tends to follow a rise in temperatures (Anisimova, 2014). In this context, the decline in the number of haemocytes may suggest a decrease the immune capacity of *T. maxima*, making it more vulnerable to infections. Due to the importance of this cellular component the overall fitness of the organism may be compromised. Moreover, the relationship with the symbionts may also be affected, as the flux of nutrients is likely to decrease, potentially contributing to the observed decline in symbiont numbers.

5.4. Biochemical analyses

In a changing environment, individual fitness is dependent on the capacity to maintain the integrity of the protein pool and cellular functions. Heat shock response (HSR), characterized by the preferential synthesis of a group of proteins - heat shock proteins (HSP's) - (Ritossa, 1962; Hofmann and Todgham, 2010) is a crucial mechanism in the acclimation process. Cellular levels of HSP tend to increase in response to unfavourable conditions, in order to raise the resilience of the organism, maintaining cellular homeostasis through the stabilization and refolding of denaturing proteins (Moseley 1997; Kregel 2002; Viant et al. 2003). The HSR is thus an important biochemical indicator to assess levels of thermal and chemical stress (Kregel, 2002; Hofmann and Todgham, 2010). The cytosolic heat shock proteins HSP70, inducible under stress, and HSC70, constitutive of the cell, are well preserved members of the HSP70 family (Ciavarra et al., 1994, Chuang et al., 2007). In the present study, however, there was no detectable effect of either stressor on the HSR pathway evaluated, as HSP70/HSC70 concentrations at the end of the experiment were quite similar across the different experimental treatments. This may indicate that the stress threshold for the activation of a HSR pathway was not reached (Feder and Hofmann, 2001). Alternatively, as the animals were exposed to the treatments for a relatively long period, it is possible that acclimation to the new conditions has been achieved. This hypothesis may be supported by evidences from previous studies, has the threshold temperature for HSP induction has been found to vary seasonally in several species, increasing in the summer months (Dietz et al., 1992; Buckley et al, 2001).

Reactive oxygen species (ROS), such as $O_2\bullet^-$, H_2O_2 and HO•, form as natural derivatives of the normal metabolism of oxygen and have important roles in cell signalling and homeostasis (Cadenas, 1989). However, during times of environmental stress, such as warmer temperatures and hypercapnic conditions, ROS levels can increase dramatically (Lesser, 2006), resulting in cellular damage and ultimately undermining of cellular functions (Cadenas, 1989). The production and accumulation of ROS, beyond the capacity of an organism to breakdown these reactive species is called oxidative stress and is considered to be an additional problem related to environmental stress (Lesser, 2006). Moreover, in photosymbiontic organims exposed to stressfull conditions, the leakage of ROS from the symbiont to the host may act as a trigger for the indution of bleaching (Lesser, 1990; Downs et al., 2002; Lesser, 2006).

Under most physiological states, ROS production tends to be matched by enzymatic antioxidant responses (Lesser, 2006) which are known to be intrinsically intertwined and dependent upon the activity of one another (Cooper et al., 2005). Superoxide dismutase

(SOD) represents the first line of defense, converting O_2^- into H_2O_2 and is followed by catalase (CAT) which removes H_2O_2 avoiding its accumulation in cells and tissues. Glutathione-S-transferase (GST), on the other hand, transforms xenobiotics into innocuous subproducts (Lesser, 2006).

In the present study, no significant differences in the enzymatic activity of both CAT and GST were found across treatments for the muscle of *T. maxima.* This can be due to the nature of the tissue analysed. Giant clams are known to accumulate SOD and other oxidative defenses on tissues from other organs, such as the gills and the siphonal mantle (which is not only directly exposed to the environment [heat and radiation] but also to the products of the symbionts' metabolic activity). The adductor muscle, on the other hand, has been found to have a comparatively small amount of SOD (Shick and Dykens, 1985). Following this, the fact that no significant responses were found across treatments in the muscle does not mean there was no oxidative stress in the organism as a whole. In fact, malondialdehyde (MDA) levels, which are commonly used as a proxy for the cellular damage caused by ROS through lipid peroxidation (Requena et al., 1996), consistently increased in *T. maxima* juveniles exposed lower a pH level.

5.5. Limitations and Further work

The results here presented constitute the preliminary findings of an ongoing work that aims to better understand how the ocean conditions foreseen for the near-future may impact giant clams. It is important to take into consideration the limitations of this first approach, such as the low and unequal sample size, which limits the statistical power of the analysis. Moreover, there is also the possibility of a misrepresentation of inter-individual variation in the different treatments, as although acquired from the same facilities there may be a significant genetic variation across individuals. Nonetheless, as previously stated, it represents a preliminary approach to the questions considered and shall be followed by a more in depth study.

More comprehensive tests regarding the effects of hypoxia, as an additional stressor, in *T. maxima* will soon take place. Moreover, the assessment of the responses to acidification and warming will continue. Growth and shell microstructure will be assessed using scanning electron microscopy (SEM). Further histological [e.g.: branchial examination; fig. S3, see preliminary results in supplementary material] and biochemical (e.g.: SOD activity, phosphate balance and Ca^+ concentrations) studies will take place, assessing additional endpoints as well as differences across tissues. More efforts regarding the effects of cli-

mate change in the photobiology of giant clams are already taking place, with the quantification of pigment contents, the evaluation of ultrastructure of *Symbiodinium* cells [through transmission electron microscopy (TEM)], and the implementation of Pulse Amplitude Modulated (PAM) fluorometry to assess the effects of the induced stressors in the photosynthetic machinery.

Through these efforts, we aim to better understand the extent of the effects of climate change related stressors in this species and trace comparisons with other giant clams and symbiont bearing organisms, such as corals. These insights may be useful to the policy and decision makers, so that the present-day management of stocks and the conservation efforts may endure the challenges that the future will bring.

5.6. Final remarks

As giant clams may live for several decades or longer (Lucas, 1988), individuals born under the present-day conditions may live long enough to experience the changes in the ocean's heat balance and chemistry, foreseen to the end of the century. Unlike animals with shorter generation times such as many coral reef invertebrates and fishes, which may have the capacity for transgenerational acclimation (Miller et al., 2012), giant clams, although highly fecund may have a reduced ability for acclimation and adaptation over the next 100 years.

Previous studies have pointed towards an alarming decrease in survival rates of *Tridacna squamosa* juveniles in response to ocean warming and acidification (Watson et al. 2012). In contrast with the results presented by Watson et al. (2012), where acidification, due to increased pCO_2 levels, appeared as the main cause of increased mortality in *T. squamosa*, temperature appears to have the most impact over *T. maxima* juveniles, regarding the endpoints evaluated. These results may, as previously emphasized by Blidberg et al. (2000), denote different responses across distinct giant clam species. Nonetheless, these differences may only result from the evaluation of distinct endpoints, highlighting the importance of multidisciplinary approaches. Ocean acidification may indeed have a preponderant impact over this group, and *T. maxima* in specific, as lower pH levels were found to increase cellular damage in the clam's tissues.

Besides their important ecological roles, these solar-powered bivalves represent an important source of income, both directly (e.g.: aquarium trade) and indirectly (e.g.: tourism) for many communities across the Indo-pacific. Therefore, the effects of ocean acidification

and climate change on these species represent a priority for further research and are of important consideration in both the management of wild populations and hatchery rearing as both are likely to be affected by the changing environment. As an example of how scientific research may help in the mitigation of climate change effects, Watson et al. (2015) was able to detect ameliorative effects of adequate light levels in the survival and growth of clams exposed to hypercapnic conditions, which mat represent an important information for conservation and production efforts in the near-future.

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6. REFERENCES

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Supplementary Material

Table S1 | Seawater parameters measured daily in the different experimental set-ups. Values (mean ± SD) were averaged across replicates over the course of the experimental period.

	28ºC		31ºC	
	8.0	7.6	8.0	7.6
	Control	*Acidification*	*Warming*	*Synergism*
Salinity	35.4 ± 1.0	35.5 ± 1.2	35.8 ± 1.1	35.8 ± 1.4
Temp (ºC)	28.1 ± 0.5	28.2 ± 0.7	31.2 ± 0.1	31.0 ± 0.8
pH	8.02 ± 0.16	7.67 ± 0.21	8.03 ± 0.06	7.67 ± 0.14

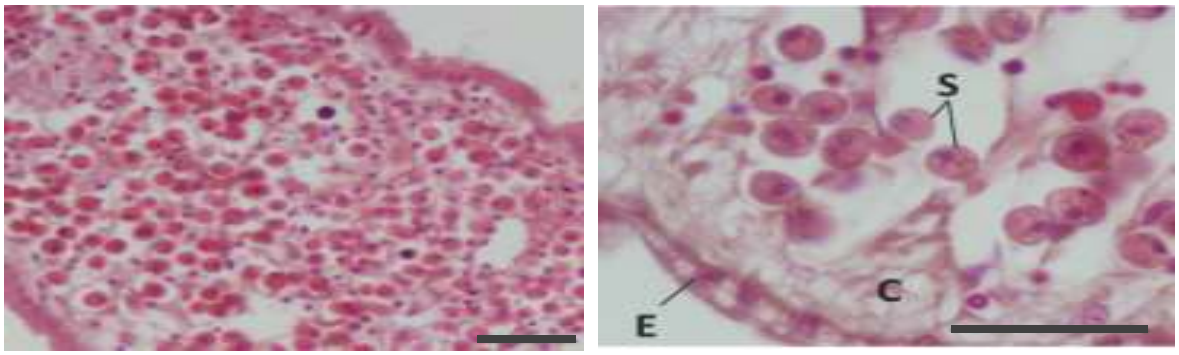

Figure S1 | Light micrographs of histological section from the siphonal mantle tissue of *T. maxima,* stained with HE. **C**, connective tissue; **E**, epithelial layer; **S**, symbiotic dinoflagellates (*Symbiodinium* spp.). SB = 50 µm.

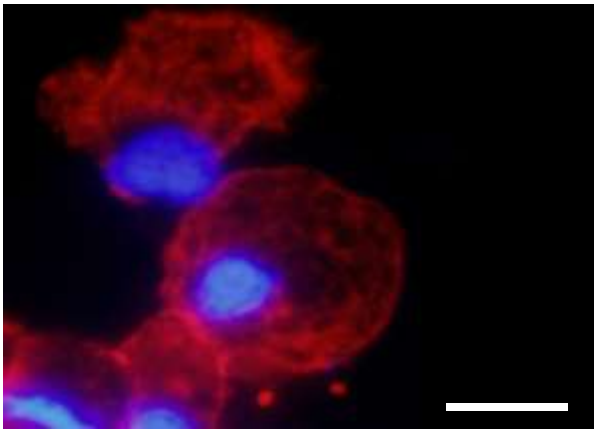

Figure S2 | Fluorescence micrographs of *T. maxima* haemocytes. SB = 10 µm.

Figure S3 | SEM micrograph of a polychaete epibiont present in the shell of *T. maxima* individual. SB = 500 µm

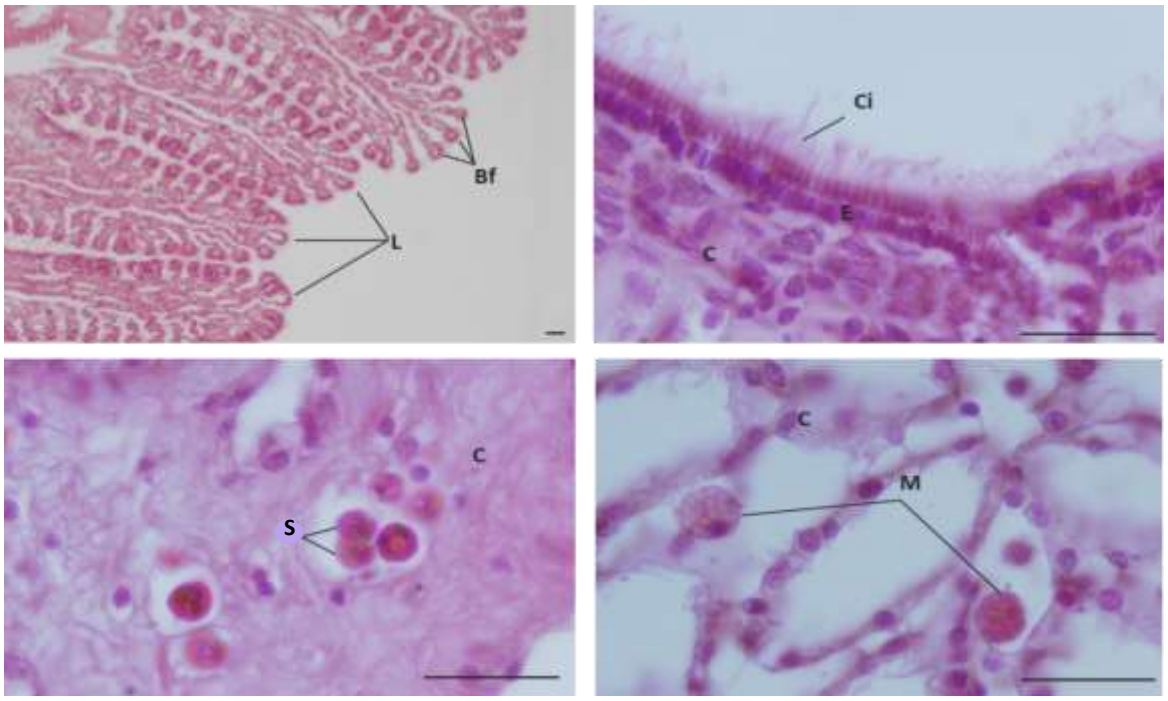

Figure S4 | Light micrographs of histological transversal cuts from branchial tissue of *Tridacna maxima,* stained with HE. **L**, Lamellae; **Bf**, Brachial filaments; **C**, connective tissue; **E**, branquial epitelium; **S**, simbiotic dinoflagellates; **M**, morulla like haemocyte. SB = 50 µm

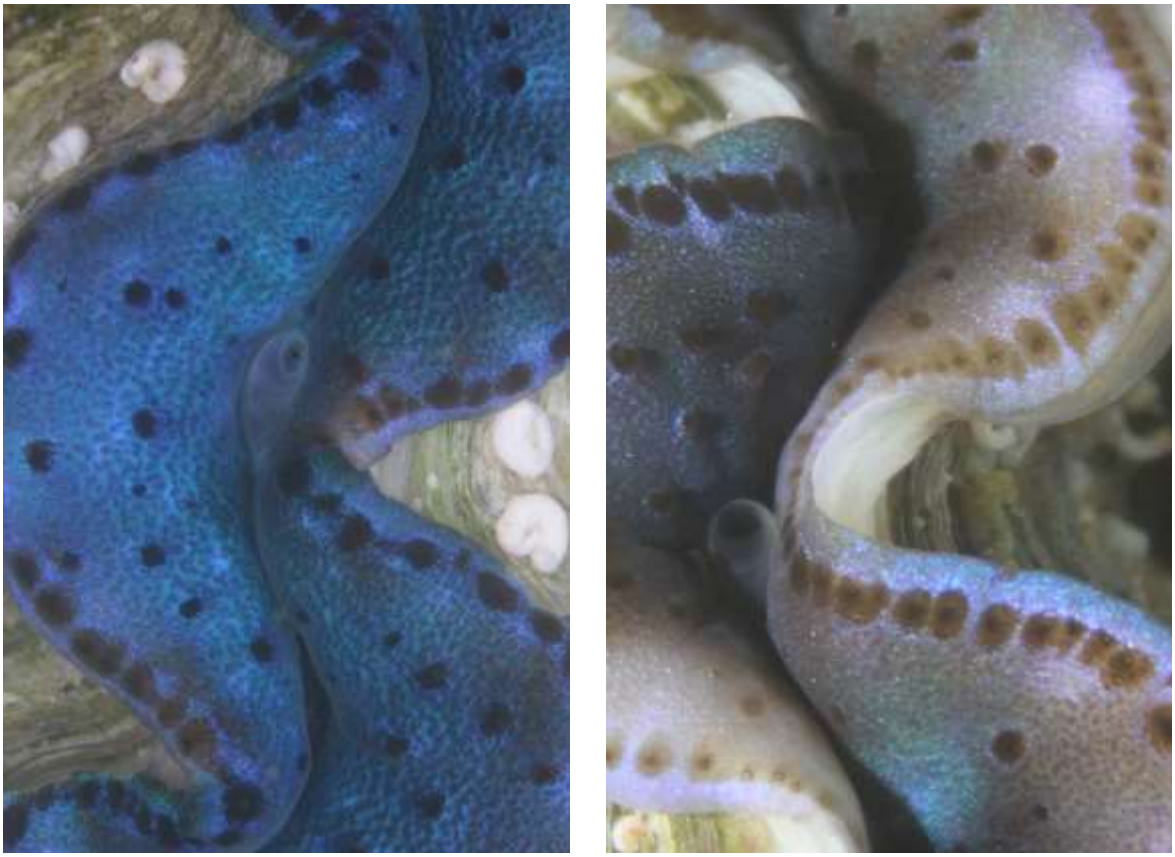

Figure S5 | Close-up contrasting the colour intensity of *T. maxima* individuals from the control (left) and the synergistic (right) treatments by the end of the exposure period.

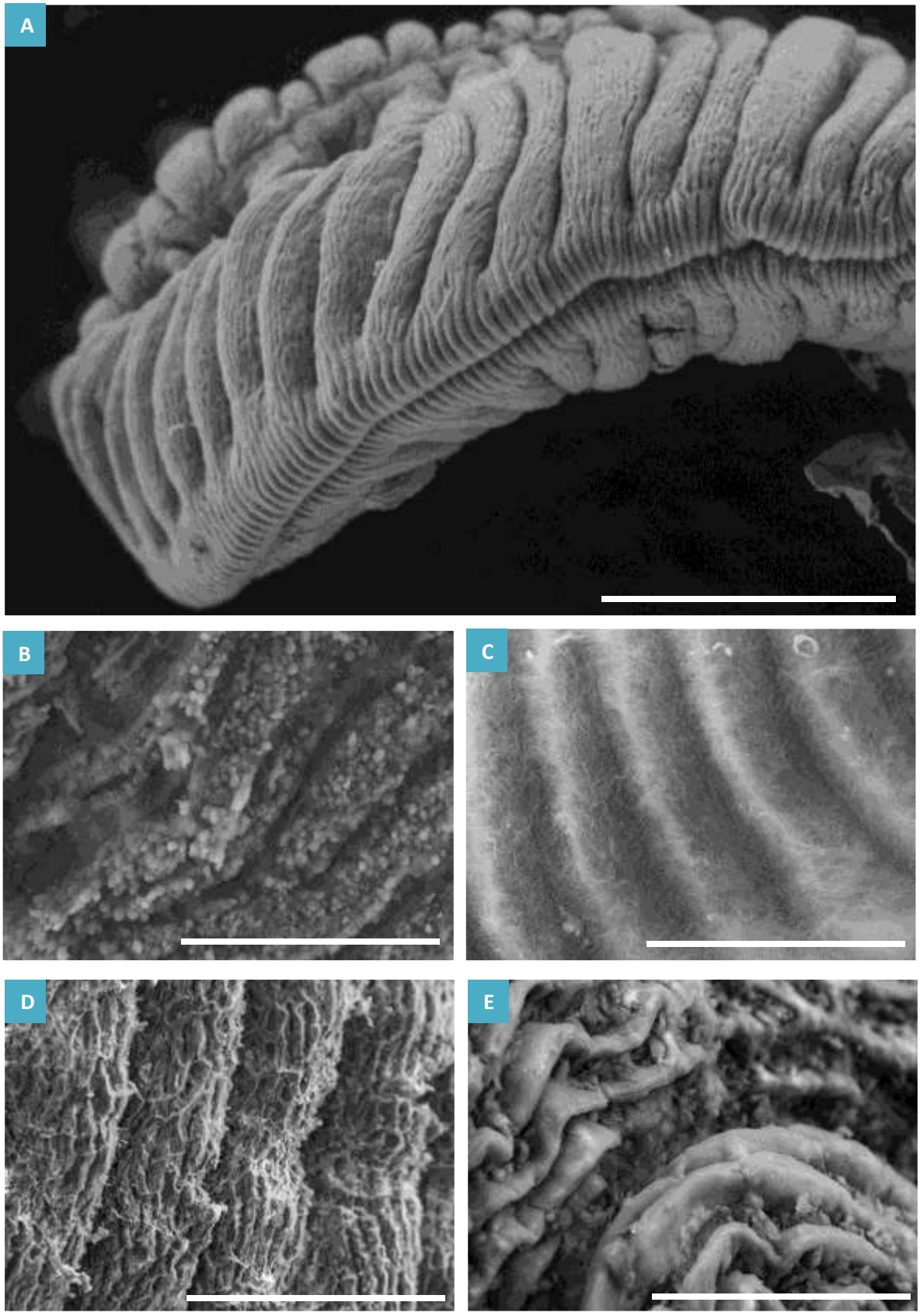

Figure S6 | SEM micrographs of *T. maxima* branchia (SB = 1 mm). **(A)** Full view of the branchia. Close-up from the **(B)** control, **(C)** warming, **(D)** acidification and **(E)** synergism (SB = 1 µm).